Physiological attributes of cold-shocked *Escherichia coli* *Salmonella* sp.p, *Staphylococcus aureus* and *Pasteurella multocida* type B.

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Preface

This work was carried out in the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, under the supervision of Professor. Sulieman Mohmmed Elsanousi.

Dedication

To my parents

To my sisters and brother

To my great friends

With my endless love

Acknowledgments

To Professor. Sulieman Mohmmed Elsanousi for his kind supervision and full support through out the research process.

All staff of the Department of Microbiology–Faculty of Veterinary Medicine – University of Khartoum for providing me all the facilities and comfort environment in order to achieve this work.

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Abstract

Injured bacteria may be difficult to detect because they fail to grow on selective media normaly used for their isolation. However, under suitable conditions injured bacteria can repair cellular damage and recover all their normal properties including virulence.

This study was conducted to observe some properties of selected cold-shocked bacteria and that included viable count of growth on selective and non- selective media before and after shock, release of ultra violet absorbing materials at 280 n.m and pathogenecity.

The bacteria studied included *Escherichia coli, Salmonella* sp.p, *Staphylococcus aureus* and *Pasteurella multocida* type B.The first three organisms were supplied by the department of microbiology- faculty of veterinary medicine - University of Khartoum. The fourth organism was supplied by the biological products department - central veterinary research laboratories center, Soba.The selective media used were MacConkey's agar for *E.coli* and *Salmonella* sp.p, Baird- Parker medium for *Staph.aureus* and *Pasteurella* medium for *Pasteurella multocida* type B. The temperature used for shocking was –20˚C (deep freezer) for 24 hours. Five rabbits of local breeds were used to study the pathogenecity of injured *Pasteurella multocida* type B.

The sensitivity of the organisms to freezing was variable; *Staph.aureus* was the most sensitive with 72.9% dead cells, 11% injured cells and 16.1% of uninjured cells followed by *Salmonella* spp, which showed 61% dead cells,18.5% injured cells and 20.5% uninjured cells and then *E.coli* which showed 55.4% dead cells ,14.8% injured cells and 29.8% uninjured cells. *Pasteurella multocida* type B, it showed great sensitivity to freezing

as it showed no colonies on the selective media but showed uncountable colonies on non-selective media.

Four rabbits injected with injured cells of *Pasteurella multocida* type B, died in 24 hours, whereas the fifth rabbit which was injected with dead cells(did not grow on the non- selective medium) did not die even after five days. The reading of the release of u.v absorbing materials by spectrophotometer at 280 n.m revealed high concentration of this materials released by *Pasteurella multocida* type B *and* less concentrations released by the three other organisms.

ملخص الأطروحة

قد يكون من الصعب الكشف عن الخلايا البكتيرية المجرحة بالبرودة لأنها لا تنمو في الأوساط الأنتقائية التي تستخدم عادة في عزلها ومع ذلك وفي ظل الظروف المناسبة يمكن لهذه الخلايا إصلاح الضرر الخلوي وإسترداد جميع خصائصها الطبيعية بما في ذلك الفوعية.

أجريت هذه الدراسة لملاحظة بعض الخصائص الفسيولوجية للخلايا المجرحة بالبرودة والتي شملت العد الحي لنموها قبل وبعد الصدمة في الأوساط الأنتقائية وغير الأنتقائية ،إطلاق مواد ممتصة بواسطة الأشعة فوق البنفسجية في درجة ٢٨٠ ن.م و إمراضية هذه الخلايا .

البكتريا التي أستخدمت في هذه الدراسة شملت الإشريكية القولونية، السالمونيلا، المكورات العنقودية الذهبية وباستورلا ملتوسيدا النوع(ب). الثلاثة انواع الاولي من البكتريا اخذت من قسم الأحياء الدقيقة- كلية الطب البيطري- جامعة الخرطوم. البكتريا الرابعة اخذت من قسم المنتجات البيولوجية- مركز المعامل والبحوث البيطرية المركزية- سوبا.

الأوساط الأنتقائية التي أستعملت شملت وسط الماكونكي لكل من الإشريكية القولونية والسالمونيلا ووسط بيرد- باركر للمكورات العنقودية الذهبية ووسط الباستورلا لباستورلا ملتوسيدا النوع(ب).

درجة الحرارة التي أستعملت لإحداث الصدمة هي٢٠ درجة تحت الصفر(تجميد عميق) لمدة ٢٤ ساعة . خمسة أرانب من الأنسال المحلية أستعملت لدراسة إمراضية خلايا باستورلا ملتوسيدا النوع(ب) المجرحة بالبرودة.

حساسية البكتريا المستخدمة في الدراسة للصدمة الباردة اختلفت من نوع لأخرفا لمكورات العنقودية الذهبية هي الأكثر حساسية حيث اظهرت نسبة ٢,٩ ٧٪ موت ونسبة ١١٪ تجريح ونسبة١ ,١٦ ٪ من الخلايا غير المجرحة تليها السالمونيلا التي اظهرت نسبة ٦١٪ موت ونسبة ١٨,٥ ٪ تجريح ونسبة٥ ,٢٠٪ من الخلايا غير المجرحة ثم الإشريكية القولونية والتي أظهرت نسبة٤ , ٥٥ ٪موت ونسبة ٤,٨ ١٪ تجريح ونسبة ٨, ٢ ٩٪ من الخلايا غير المجرحة.

باستورلا ملتوسيدا النوع(ب) أظهرت حساسية كبيرة للتجميد في درجة ٢٠ تحت الصفر حيث لم تظهر أي مستعمرات في الوسط الأنتقائي بينما ظهرت مستعمرات غير قابلة للعد في الوسط غير الأنتقائي.

الأرانب الاربعة التي حقنت بخلايا باستورلا ملتوسيدا النوع(ب) المجرحة بالبرودة ماتت في خلال ٢٤ ساعة ، أما الأرنب الخامس الذي حقن بخلايا ميتة (لم تظهر مستعمرات في الوسط غير الأنتقائي) لم يمت حتي بعد خمسة أيام. أظهرت القراءة بواسطة جهاز القياس الطيفي في ٢٨٠ نانومتر إطلاق مواد ممتصة للأشعة فوق البنفسجية بتركيز عالي بواسطة خلايا باستورلا ملتوسيدا النوع(ب) وبتركيز أقل بواسطة الانواع الثلاث الاخري من البكتريا .

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CHAPTER ONE

Introduction

Food may contain a variety of micro-organisms such as *Salmonella, E.coli*, yeasts and moulds. The presence of microorganisms in food stuffs can affect both the safety and quality of the product. Consequently, food manufacturers have developed food processing treatments that help preserve foods, by destroying or injuring micro-organisms that are present in food and thus preventing their growth.

There are many sites within a bacterial cell that can become damaged when the bacteria are subjected to these food processing treatments. These sites include the genetic material of the cell (DNA, RNA) and also the cell membrane. Some bacteria have developed ways to survive some processing treatments. These include the production of heat shock and cold shock proteins that help the cell function normally under higher or lower temperatures than normal.

Some treatments will cause irrepairable damage and the cells will be destroyed. However, sometimes the damage will be repairable and the cells are able to recover. Micro-organisms that are destroyed by processing will not cause subsequent food poisoning or spoilage, but injured and repaired organisms can cause subsequent food spoilage or poisoning.

The uninjured cells will be those organisms that are most easily detected and enumerated by current microbiological methods. The results gained from use of these methods are used to assess the risks of food spoilage and safety. However, the injured bacteria must also be counted because these organisms can also pose a food safety and/or spoilage risk as they can repair if the

conditions become favorable. Therefore suitable test methods to detect injured bacteria within foodstuffs should be developed (Linda, 2001).

Despite the great benefit of freezing to food industries, a number of workers have observed that many pathogenic bacteria survived prolonged storage in various kinds of frozen food. (Macfadyen ,1990) found that, after freezing at –190˚C for 20 hr, bacterial cultures not only survived, but also retained their biochemical and other characteristics behaviors.The existence of injured microorganisms in food and their recovery during culturing procedures is critical. Microbial injury is characterized by the capability of a microorganism to return to normality during a resuscitation process in which the damaged essential components are repaired (Ahn *et al*; 1964). Injured microorganisms present a potential threat in food safety since they may repair themselves under suitable conditions. Detection of injured microorganisms can be important to practical interpretations of data in food microbiology (Vivian, 2008).

Many types of bacteria are associated with food-borne diseases all over the world e.g. *E. coli*, *Salmonella spp*., and *Staphylococcus aureus.*

E. coli:

Since 1885, when it was first isolated from children's faeces and described by the German bacteriologist Theodor Escherich, scientific attention has been lavished on *Escherichia coli* to such an extent that it is today probably the best understood free–living organisms. Strains of *E.coli* were first recognized as a cause of gastroenteritis by workers in England investigating summer diarrhoea in infants in the early 1940s. Until 1982, strains producing diarrhoea were classified in to three types based on their virulence properties, enteropathogenic *E.coli*(EPEC), enteroinvasive *E.coli(*EIEC),

enterotoxigenic *E.coli(*ETEC). They are not very common causes of food borne illness in developed countries, but an important cause of childhood diarrhoea in less developed countries. *(*ETEC) is also frequently associated with so called traveller's diarrhoea. However since 1982 enteroheamorrhagic *E.coli*(EHEC) particulary associated with serotypes 0157:H7 has been recognized as the cause of a number of out breaks of heamorrhagic colitis and heamolytic uremic syndrome, particularly in north America where food such as under- cooked ground meat , raw milk and fresh product have been implicated. (Adams and Moss, 2008).

Salmonella spp:

Adams and Moss (2008) also recorded that most *Salmonellae* are regarded as human pathogens, though they differ in the characteristic and the severity of the illness they cause. Typhoid fever is the most severe and consequently was the earliest infection to be reliably described .Salmonellosis is described as a zoonotic infection since the major source of human illness is infected animals. Transmission is by the feacal- oral route, where by intestinal contents from an infected animal are ingested with food or water. A period of temperature abuse which allows the salmonella to grow in the food and an adequate or absent final heat treatments are common factors contributing to outbreaks. Meats, milk, poultry and eggs are primary vehicles, they may be undercooked, allowing the salmonellas to survive or they may cross-contaminated other food that are consumed with out further cooking. Human carriers are generally, less important in the transmission of salmonellosis.

***Staphylococcus aureus*:**

They were first described by the Scottish surgeon, Sir Alexander Ogston as the cause of a number pyogenic (pus forming) infections in human. The presence of small numbers of *Staph. aureus* in food is not uncommon . It

will occur naturally in poultry and other raw meats as a frequent competent of the skin flora. There however been out breaks caused by milk products such as dried milk and chocolate milk, where growth and enterotoxins productions occurred in the raw milk and the enterotoxin, but not the organism, survive subsequent pasteuralization .Contamination by food handlers is also probably a frequent occurrence in view of the high rate of human carriage. (Adams and Moss, 2008).

Pasteurella multocida:

Pasteurella multocida is wide spread pathogen in the animal kingdom but also in human .It causes lives- threatening illnesses of rabbits, cattle, sheep and birds. Although *Pasteurella multocida* is an important pathogen it could occur as acommensal of the normal naso- pharyngeal micro flora of adult animals e.g. cattle , rabbits , cats and dogs The species *Pasteurella multocida* consists of four subspecies multocida , gallicidia, septica and tigiris . *Pasteurella multocida ssp multocida* in return is divided in to serogroups (A, B, D, E and F), as cited by, (Harper *et al*; (2006) and Capitini *et al*; (2002). *Pasteurella multocida* toxin (PMT) is the major pathogenic determinant of *Pasteurella multocida.* Rabbits are highly susceptible to infection with *Pasteurella multocida.* (Proft, 2009).

Objectives:

The objectives of this study were:

1. To study the effect of freezing in viable count of *E. coli*, *Salmonella spp*, *Staphylococcus aureus* and *Pasteurella multocida* type B.
2. To measure the release of U.V absorbing materials from these freeze-injured organisms at 280 n.m.
3. To study the pathogenecity of freeze-injured *Pasteurella multocida* type B.

CHAPTER TWO
LITREATURE REVIEW

2.1. Injury to microorganisms:

Disinfection or sterilization treatment by heating, irradiation, or chemicals can cause injury to microorganisms at sub lethal levels. Microbial injury is the inability to grow under conditions suitable for the uninjured microorganisms. This inability of injured microorganisms to grow is explained in terms of more complex or different nutritional requirements or in terms of increased sensitivity to environmental conditions such as incubation conditions (time or temperature) or to chemical agents such as halogen compounds. (Hideharu, 2006)

2.2. Definition of injured cells:

There are many definitions that described the injured cells, one of these definitions was by Clarck and Ordal (1969) who defined the injured cells are those cells which can form colonies on enriched media but can not form colonies on stressing media

But according to (Hideharu,2006) who goes further to say that injured microorganisms can be distinguished from those that are dead or mutated by their ability to regain normal physiological activity when placed in appropriate conditions for cultivation. The return to normal physiological function has been termed repair. The extent and severity of sub lethal injury, the mechanisms of injury, and the mechanisms and degree of recovery vary with the sterilization procedures, the species, the strains, the condition of the microorganism, and the methods of repair. Injury to spore formers has been detected at different stages of the spore cycle. The sites of injury include

damage to enzymes, membrane disruption, and/or damage to DNA or RNA. Information on the sub lethal injury and recovery of microorganisms is very important in evaluating sterilization/disinfection procedures.

2.3. Causes of injury:

There are so many causes of injury including sub lethal heat, freezing, freeze-drying, drying, irradiation, high hydrostatic pressure, aerosolization, dyes, sodium azide, salts, heavy metals, antibiotics, essential oils, sanitizing compounds, and other chemicals or natural antimicrobial compounds (Vivian,2008).

2.3. a. irradiation:

Cells irradiated in suspension with dosages sufficient to render them completely nonviable on culture continued to respire on lactic acid at about 60% the rate of non-irradiated cells, but the residual activities on the other substrates tested were less than 10% to 12%. Lyophilized cells irradiated with sufficient dosages for cultural non-viability retained 40 to 80% of the oxidative capacity of non-irradiated cells on the test substrates. Cells lyophilized and irradiated in the presence of albumin generally retained a greater portion of their oxidative activities compared with cells lyophilized and irradiated in buffer. Cells irradiated in the presence of various oxidizable substrates gave varying results, depending both on the substrate present during irradiation and the substrate added for the oxidative studies. (Ahn, *et al;* 1964).

2.3. b. The pH:

Chaveerach, *et al;* (2003) studied the culturability of 10 strains of *Campylobacter jejuni* and *Campylobacter coli* after the bacteria were exposed to acid conditions for various periods of time. *Campylobacter* cells could not survive 2 hr under acid conditions (formic acid at pH 4). The 10

Campylobacter strains could not be recovered, even when enrichment media were used. The treated bacteria changed into a viable but nonculturable (VBNC) form.

Sato, *et al;* (1972) concluded that the cells of *Escherichia coli* at the logarithmic phase of growth lost their colony-forming ability when the cells were suspended in Tris buffer containing 0.15M sodium chloride. The colony-forming ability was recovered rapidly when sodium chloride-treated cells were incubated in Tris buffer containing magnesium ion immediately after the treatment. The magnesium- mediated recovery was not inhibited by chloramphenicol 2, 4-dinitrophenol, or sodium azide. Moreover, a higher recovery was observed by incubation at 3°C rather than at 30°C. A significant release of UV-absorbing materials occurred during incubation in Tris buffer containing sodium chloride. This release was almost completely suppressed when magnesium ion was present in the incubation medium.

2.3. C.Effect of temperature:

2.3. C.1.The heat:

When Wilson and Davies (2008), exposed *Salmonella senftenberg* 4969 to sub lethal heating in phosphate buffer, pH 7·0, at 52°C, they reported , thermally injured cells were obtained and characterized by their relative inability to form colonies on trypticase soy yeast extract agar compared to minimal medium (M9) agar. During subsequent incubation at 37°C in liquid media, more injured cells were capable of repair in M9 than in nutrient media used for pre-enrichment purposes.

A similar results obtained by Tomlins and Ordal (1971) through the heating of *Salmonella typhimurium 7136* at 48°C for 30 min, and that produced a population of cells that were incompetent at division on Levine Eosin Methylene Blue Agar containing 2.0% NaCl (EMB-NaCl). When these

injured cells were placed in fresh citrate salts medium they recovered, and regained their tolerance to the EMB-NaCl medium and grew out.

Incubation of heat-injured *Listeria monocytogenes* cells in broth or chicken slurry at 5ºC (cold-enrichment) did not allow repair of potentially lethal injury i.e. it did not allow recovery of cells that would otherwise have died if incubated at a higher temperature. In some cases incubation of heat-injured cells at 5ºC resulted in death of a proportion of the population. (Mackey, *et al*; (1994).

2.3. C.2. Cold shock (Phoenix phenomenon):

The effect of cold diluent on viability was first reported by Sherman and Albus (1923) who reported that c 95% of cells were killed within 1hr.when the temperature of a 4 hr. culture of *E.coli* was reduced suddenly from 45°C to 10°C, while no similar loss in viability was observed in 12 day old cultures.

A loss in viability also was observed by Sherman and Cameron (1934) upon transferring *E.coli* in the log phase from a medium at 45°C to a medium at 10°C .*Pseudomonas* was shown to be sensitive to cold shock in the log phase by Gorril and McNeil (1960). In contrast, MacKelvie, Gronlund and Campbell (1968) found that stationary phase cultures of *Pseudomona aeruginosa* were susceptible to cold shock during harvesting but log-phase cells were not. The same phenomenon i.e cold-shock of log phase cells was observed in *Aerobacter aerogenes* (Strange and Dark, 1962; Strange and Ness, 1963) but not in *Staphylococcus aureus* (Gorril and McNeil, 1960) or in *Streptococcus hydrogenans* (Ring 1965a). Williams (1956) observed a similar fall in viable count with log phase cells of thermoduric bacterium (Micrococcus 9 ,dairy Group III B) in glucose broth at 37.5°C , but since it never occurred when cultures were incubated at 15°C or 25°C, he concluded

that the effect was due solely to the temperature of incubation. The more probable reason for failure to observe the phenomenon was the small difference between the incubation temperature (i.e 15°C and 25°C) and the temperature of the diluents, assuming the latter was at room temperature.

The term (cold- shock) or (the effect of sudden freezing on bacterial cells) was first used by Elsanousi (1975) instead of (Phoenix phenomenon) and other investigators such as (Traci and Duncan, 1974); (Shoemaker and Pierson, 1976) also used this term.

Despite the fact that, Gram – negative organisms are more susceptible to freezing than Gram – positives, ElSanousi (1975) reported that cold–shock phenomenon which results in loss of viability in cultures of Gram- negative bacteria, also occurred in cultures of *Clostridium perfringens*. Cells at different stages of growth were subjected to cold diluents for 1hr. Cells at all stages of growth were inactivated by cold diluents .Those from the early logarithmic phase were most sensitive , viability falling by 60% over 1hr. most of which (c90%) occurred in the first 15 minutes. Also the "Phoenix phenomenon" was observed with *Clostridium perfringens* Hobbs' serological type 9 (HT9) in a cooked meat medium at 81.7ºC by a decrease in plate count (phase I), followed by an increase in count to the initial level (phase II) and a continued increase above the initial count (phase III).

This phenomenon was reproduced in experiments with sporulation-negative mutants derived from HT9 inocula of various cell ages, and different assay media (sulfite-iron agar, tryptose-soytone-yeast extract agar, prereduced peptone-yeast extract agar, prereduced veal agar, and veal agar). When strict anaerobic conditions were employed, it was necessary to increase the heating temperature to 52.3ºC to observe the phenomenon. The phenomenon

was eliminated at 52.3ºC when a combination of strict anaerobic conditions, prereduced media, and prereduced veal diluent was employed.

The addition of nalidixic acid at the minimum point of the growth curve (end of phase I) had no effect on the appearance of phase II; however, phase III was completely inhibited. This indicated that phase I and II were an injury-recovery process (Shoemaker and Pierson, 1976).

Several observations have been made in regard to cold shock lethality of *Clostridium perfringens*: (i) loss of viability was not consequence of exposure of the cells to air; (ii) stationary-phase cells were much more resistant to cold shock at 4°C than exponential-phase cells; (iii) at 4 °C 96% of an initial population of exponential-phase cells was killed upon cold shock and 95% of the remaining population was killed within 90 min of continued exposure at 4°C (iv) the minimal temperature differential for detectable cold shock lethality was between 17°C and 23°C, and the maximum beyond which lethality was not appreciably increased was between 28°C and 33°C. Up to 75% of viable cold-shocked cells were injured, as demonstrated by cold shocking late exponential-phase cells at 10°C and using differential plating procedure for recovery.

Repair of injury was temperature dependent, and occurred in a complex medium and 0.1% peptone but not water. Nalidixic acid, chloramphenicol, and rifampin did not inhibit repair of injury (Traci and. Duncan, 1974).

Campylobacter jejuni is fastidious in terms of their temperature requirements, being unable to grow below ca. 31°C, but have been found to be physiologically active at lower temperatures and to tolerate exposure to low temperatures in a strain-dependent manner .Chan *et al;* (2001) studied nineteen field isolates of *C. jejuni* (10 of clinical and 9 of poultry origin) for their ability to tolerate prolonged exposure to low temperature (4°C).

Although substantial variability was found among different strains, clinical isolates tended to be significantly more likely to remain viable following cold exposure than poultry-derived strains.

In contrast, the relative degree of tolerance of the bacteria to freezing at–20°C and freeze-thawing was strain specific but independent of strain source (poultry versus clinical) and degree of cold (4°C) tolerance.

Also Klein *et al* ;(1999) noted that *Bacillus subtilis* has developed sophisticated mechanisms to withstand fluctuations in temperature. Membrane fatty acids are the major determinants for a sufficiently fluid membrane state to ensure the membrane's function at all temperatures. The fatty acid profile of *B. subtilis* is characterized by a high content of branched fatty acids irrespective of the growth medium.

When Oliver **(**1981) conducted a study on the survival of *Vibrio vulnificus*, an estuarine human pathogen, in oyster homogenates held at 4˚C. he reported a rapid and dramatic decrease in viability not attributable to either cold shock or the oyster homogenate alone but to a combination of the two. Such a decline was not observed with *Vibrio parahaemolyticus.*

Also the results showed that chilled *V. vulnificus* cells were unable to repair themselves in brain heart infusion broth at 37˚C. *V.vulnificus* cells incubated on whole raw oysters at 0.5˚C also exhibited a decline in viability, but of a lesser degree.

When *Campylobacter jejuni* cultures that had been grown in broth at 39°C were subcultured into fresh medium at 30°C, there was a transient period of growth followed by a decline in viable-cell numbers before growth resumed once more (Vivian, 2008).

When lactic acid bacterial cultures were frozen at −20°C for 24 hr, the cell viability decreased drastically, but when they were cold shocked at 10°C for

2 hr. prior to freezing, viability improved significantly for the *Lactococcus lactis* subsp. *lactis* strains (25–37%) and *Pediococcus pentosaceus* PO2 (18%), but not for the *Lactococcus lactis* subsp. *cremoris* strains tested or for one strain of *Lactobacillus helveticus* LB1 and *Streptococcus thermophilus* TS2. (Kim and Dunn, 1997)

2.3. C.3. Effect of chilling:

As found by earlier workers**,** *Escherichia coli* growing in broth at 37°C was rendered incapable of gross multiplication either on nutrient agar or in nutrient broth by sudden cooling in many diluents at 4°C. Killing was due to the joint action of suitable diluents and of sudden chilling. Organisms in the stationary phase of growth were completely resistant.

The susceptibility of growing organisms to sudden chilling changed rapidly during the exponential phase. Comparison with the survival after exposure to streptomycin, another bactericidal agent which has no effect on stationary phase cultures, showed that survival after chilling was not due to a fraction of the population being in the stationary phase. (Meynell, 1958).

Smith (2006) investigated the effect of chilling and freezing strains of *Escherichia coli* and *Salmonella* serotypes in nutrient broth, a non-inhibitory liquid medium. The phase of growth, small changes in composition of test medium, and sub-cultures made after primary isolation, influenced survival. Therefore, such influences must be considered when attempting to extrapolate results from pure cultures on laboratory media, to predict behavior of similar organisms in foods during chilling and freezing.

Foods associated with *Clostridium perfringens* outbreaks are usually abused after cooking. Because of their short generation times, *C. perfringens* spores and cells can grow out to high levels during improper cooling. (De Jong *et al*; 2004).

2.3. C.4. Effect of freezing:

The effect of different types of freezing on bacteria was studied by many workers. These studies included the effect of repeated freezing and thawing, frozen storage for various periods of time, the types of freezing (slow or fast), and the rate of death of bacteria due to freezing (rapid or slow).

Freezing and thawing of *Escherichia coli* has two types of lethal effect, (i) immediate, i.e. that occurring during freezing at −20°C or −196°C and immediate thawing, and (ii) delayed, i.e. that occurring during frozen (−20°C) storage. On this basis, Alur and Grecz (1975) concluded that cell death of *E. coli* by freezing, cold storage, and thawing is due to DNA degradation and loss of its vital integrity.p

The acquired freeze-thaw tolerance was investigated for *Lactococcus lactis* spp *.diacetylactis*. Pretreatment of microorganisms at less severe temperatures to initiate cold tolerance gave *L. lactis* spp.*diacetylactis* improved cell viability after successive freezing and thawing.

The ability of cells to survive freeze-thaw was dependent on factors experienced prior to freezing. Factors affecting lactic acid bacteria survival during freeze-thaw cycles were found to be different diluents, growth phase, and different cold temperatures (Lee *et al*; 1976).

Also metabolic injury was noted by Moss and Speck (1966) when *Escherichia coli* stored at –20°C and resulted in non lethal or "metabolic" injury to a proportion of the surviving population. The injury was manifested as an increased nutritional requirement after freezing. Injured cells could not grow on a minimal agar medium, but could develop on Trypticase soy agar. The percentage of injured survivors varied among strains, but was little affected by altering the freezing menstruum.

Freezing produces its effect whether the cells are frozen on the growing media or stored in a freeze dried - state. On the other hand, keeping the freeze- dried cells at –20°C resulted in a marked percentage of injured cells and this may lead to false judgment when enumeration of the actual number of the cells is attempted in a selective media. This fact ought to be given some consideration when dried food is to be subjected for bacteriological examinations (Layla, 1987).

A mixed inoculum of *Salmonella derby* or *Salmonella typhimurium* and *Escherichia coli* was injected into the intestinal region of Pacific oysters (Crassostrea gigas) which were then frozen by four methods. Both species of *Salmonella* proved to be highly sensitive to freezing, regardless of the freezing method, and showed a survival of 1% or less after 48 hr. *E. coli* proved less sensitive, showing a wide and capricious variability of survival during the first week of storage, with survival ranging from 10% to 30% (Digirolamo *et al* ;2006).

Bacteria isolated from processed meat that subjected to freezing at –20°C to study the effect of freezing on bacterial cells, freezing was found to produce injury in bacterial cells. Consequently , freeze- injured cells manifested a) an extended lag phase b) an increased sensitivity to selective agents incorporated in selective media c) in addition ,*Staph. aureus* lost their salt tolerance.(Fatima ,1991).

Layla, (1987) reported that the freezing of *Staphylococcus. aureus* , *E. coli, Clostridium perfringens* and *Pseudomonas aeruginosa* in meat was less harmful when compared with freezing done in the laboratory media to the same temperature (–20°C) and the same period of time(24 hr).

2.4. Colony forming ability:

Many attempts have been made to study the effect of cold shock in the bacterial cell and its ability to form colonies.

Rodney *et al*;(1973) found that the exposure of exponentially growing *Pseudomonas fluorescens* P7 cells to heating at 36°C for 2 hr. in a defined medium, followed by cooling to 25°C and further incubation at this temperature, the optimal growth temperature, resulted in the apparent death of approximately 99% of the cells, as determined by their inability to form colonies on Trypticase soy agar. Continued incubation at 25°C resulted in an extremely rapid increase in the Trypticase soy agar count, demonstrating that the phenomenon observed was not death but rather injury.

Speck *et al* ;(1973) demonstrated that when two strains of *Escherichia coli* are being frozen in water or sterile foods at –20°C, they manifested death and repairable injury. The injured survivors were inhibited from forming colonies on violet red bile agar (VRBA) or deoxycholate lactose agar; this inhibition was greater when enumeration was done by the pour plate method instead of the surface or surface-overlay method. Injured cells repaired rapidly in Trypticase soy broth (TSB), and the repair was about maximum after 1 hour at 25°C.

Fast freezing and slow thawing of *Salmonella anatum* cells suspended in water resulted in injury of more than 90% of the cells that survived the treatment. The injured cells failed to form colonies on the selective medium (xylose lysine-peptone agar with 0.2% sodium deoxycholate) but did form colonies on a non selective (xylose-lysine-peptone-agar) plating medium.

The repair process might involve energy metabolism in the form of adenosine triphosphate.The freeze-injured cells were highly sensitive to lysozyme, whereas unfrozen fresh cells were not. In the presence of

phosphate or minimal broth this sensitivity was greatly reduced. This suggested that, at least in some of the cells, the injury involved the lipopolysaccharide of the cell wall and adenosine triphosphate synthesis was required for repair. (Ray, *et al*; 1972).

2.5. Viable but- nonculturable (VBNC) state:

In microbiology the terms 'viability' and 'culturability' are often equated. However, in recent years the apparently self-contradictory expression 'viable-but-nonculturable' ('VBNC') has been applied to cells with various and often poorly defined physiological attributes but which, nonetheless, could not be cultured by methods normally appropriate to the organism concerned. These attributes include apparent cell integrity, the possession of some form of measurable cellular activity and the apparent capacity to regain culturability. (Douglas, *et al*; 1998).

The recognition of sources and reservoirs of bacterial pathogens is a central issue in the control of infectious diseases. Until the 1980s this was achieved by isolation of the pathogen concerned from appropriate sample material. Two developments, the establishment of molecular detection methodologies and the proposal that some bacteria may enter a 'viable but nonculturable' (VBNC) state, require us to re-evaluate this approach. Molecular detection methods can reveal the presence of nucleotide sequences or antigens in diverse sample materials. (Smith, 2002).

A short-term response to cold shock is necessary for bacteria to transit to a viable but nonculturable state and/or for their physiological and genetic adaptation to psychrotrophic life.

It is emphasized that cell responses to cold and heat shocks are different and that DNA dynamics (its supercoiling, multiplebending, and condensation) and the rearrangement of the protein-synthesizing apparatus of cells

(including the induction of alternative translational mechanisms) may play a central role in cell response to cold shock. The role of molecular chaperones in cold shock response is presumably of less importance than it is in the case of heat shock (Golovlev, 2003).

2.6. Cold shock genes:

Various species and genera of bacteria show the same responses to low temperatures. Cold induces cold shock genes, by causing significant changes in the regulation of protein synthesis. The synthesis of major proteins in the microbial cell is suppressed. However, there is synthesis of a great deal of new proteins, the so-called cold shock ones. The chief protein in this family is *E. coli* CspA that activates the translation of other cold shock genes and negatively regulates the expression of its own gene. *E. coli* CspA homologies were identified in many bacteria. They can be also identified in other microorganisms, including the pathogens of infectious diseases. This can be attributable to the presence of common antigens in different bacteria (Basnak'ian, 2001).

Responses of cyanobacterial cells to low-temperature stress are basically of two types. One type involves the cold-induced desaturation of fatty acids in membrane lipids such that the membranes become more fluid to compensate for the decrease in membrane fluidity that would otherwise occur at the temperature .The other type involves the low-temperature-induced synthesis of enzymes that enhance the efficiency of transcription and translation to compensate for the decrease in the efficiency of these processes at low temperature. Both types of response serve to protect the cyanobacterial cells from the detrimental effects of low-temperature stress (Los and Murata, 2000).

Also Yamanaka and Inouye (2001) found that the synthesis of CspA, the major cold-shock protein of *Escherichia coli*, is dramatically induced upon cold shock. It was recently reported that there is massive presence of CspA under nonstress conditions, and it is thus claimed that CspA as the cold-shock protein is a misnomer.

They re-examined and confirmed that CspA is induced upon culture dilution at 37 ˚C. However, its induction level is one-sixth of the cold-shock-induced level, clearly indicating that the major stress that induces CspA is cold shock.

It was further found that CspA induction can be achieved not only by culture dilution but also by the simple addition of nutrients, and that it was almost completely abolished in the presence of rifampicin or nalidixic acid. Nutritional upshift causes the induction of only CspA but not other cold-shock-inducible CspA homologues. The amount of CspA mRNA rapidly and transiently increased by culture dilution, but its stability was not significantly changed. These results suggest that CspA is a nutritional-upshift stress protein as well as a cold-shock stress protein, and that CspA induction following nutritional upshift may be due to transcriptional activation.

In *Escherichia coli*, the CspA family includes nine homologous proteins, CspA to CspI. Four of these proteins are transiently and significantly induced upon temperature downshift from 37°C to 15°C (hence the name Csp, for Cold Shock Protein). None of the CspA homologues appears to be singularly responsible for cold-shock adaptation, since individual Csp genes are dispensable at both normal and low growth temperatures. However, while all single, double or triple deletion mutants of Csp genes grow at low temperature, a quadruple deletion strain (CspA ,CspB ,CspG ,CspE) is cold-

sensitive for growth, indicating that a presence of at least one of these CspA family proteins is required to support growth at low temperature. Overproduction of any one of the nine CspA family genes, with the exception of CspD, complements the cold-sensitivity of the quadruple deletion strain, indicating that the functions of these proteins are redundant (Phadtare and Severinov, 2005).

The food borne pathogen *Listeria monocytogenes* has many physiological adaptations that enable survival under a wide range of environmental conditions. The microbes overcome various types of stress, including the cold stress associated with low temperatures in food production and storage environments. Cold stress adaptation mechanisms are therefore an important attribute of *L. monocytogenes*, enabling these food pathogens to survive and proliferate to reach minimal infectious levels on refrigerated foods.

This phenomenon is a function of many molecular adaptation mechanisms. Therefore, an improved understanding of how cold stress is sensed and adaptation measures implemented by *L. monocytogenes* may facilitate the development of better ways of controlling these pathogens in food and related environments. Research over the past few years has highlighted some of the molecular aspects of cellular mechanisms behind cold stress adaptation in *L. monocytogenes* .

Listeria monocytogenes possesses three small, highly homologous protein members of the cold shock protein (Csp) family. Stephan (2006) used gene expression analysis and a set of mutants with single, double, and triple deletions of the Csp genes to evaluate the roles of CspA, CspB, and CspD in the cold and osmotic (NaCl) stress adaptation responses of *L. monocytogenes*. All three Csps are dispensable for growth at optimal temperature (37 ˚C).

These proteins are required for efficient cold and osmotic stress tolerance of this bacterium. The hierarchies of their functional importance differ, depending on the environmental stress conditions: CspA,CspD,CspB in response to cold stress versus CspD,CspA/CspB in response to NaCl salt osmotic stress.

The fact that Csps are promoting *L. monocytogenes* adaptation against both cold and NaCl stress has significant implications in view of practical food microbial control measures. The combined or sequential exposure of *L. monocytogenes* cells to these two stresses in food environments might inadvertently induce cross-protection responses.

2.7. Cold shock proteins:

All organisms examined to date, respond to a sudden change in environmental temperature with a specific cascade of adaptation reactions that, in some cases, have been identified and monitored at the molecular level. According to the type of temperature change, this response has been termed heat shock response (HSR) or cold shock response (CSR).

During the HSR, a specialized sigma factor has been shown to play a central regulatory role in controlling expression of genes predominantly required to cope with heat-induced alteration of protein conformation. In contrast, after cold shock, nucleic acid structure and proteins interacting with the biological information molecules DNA and RNA appear to play a major cellular role. Currently, no cold-specific sigma factor has been identified. Therefore, unlike the HSR, the CSR appears to be organized as a complex stimulon rather than resembling a regulon.

Special emphasis is placed on recent findings concerning the nucleic acid binding cold shock proteins, which play a fundamental role, not only during

cold shock adaptation but also under optimal growth conditions. (Weber and Marahiel, 2002).

The cold-shock response of *Escherichia coli* describes a specific pattern of gene expression in response to abrupt shifts to lower temperatures. This pattern includes the induction of cold-shock proteins, synthesis of proteins involved in transcription and translation, and repression of heat-shock proteins. The identified cold-shock proteins are involved in various cellular functions from supercoiling of DNA to initiation of translation.

The major cold-shock protein, CspA, has high sequence similarity with three other *E.coli* proteins ,CspB, CspC, and CspD. Using translational lacZ fusions, CspB was found to be cold-shock inducible at the level of transcription like CspA, while CspC and CspD were not.

The Csp proteins, which share sequence similarity with other prokaryotic proteins and with the 'cold-shock domain' of eukaryotic Y-box proteins, may have a function in activating transcription or unwinding or masking RNA molecules. Because the cold-shock response can also be induced by the addition of certain inhibitors of translation, it has been proposed that the state of the ribosome is the physiological sensor for the induction. In addition to E. coli, cold-shock proteins have also been found in other prokaryotic and eukaryotic organisms (Inouyem, 1994).

According to Graumann and Marahiel (1996) bacteria respond to an abrupt decrease in temperature with a specific response, in which cold-induced proteins (CIPs) are transiently expressed at a higher level. Employing two-dimensional gel electrophoresis, several CIPs have been identified. In spite of this, the overall function of the cold shock response is unclear. Recently, the main attention has focused on a group of conserved cold shock proteins (CSPs) that have been shown to have the highest induction after cold shock

and to play a major regulatory role in the physiology of adaptation to low temperatures.

CSPs, of which *Escherichia coli, Bacillus subtilis,* and *Bacillus cereus* possess a family comprising at least 3-7 proteins, are small acidic proteins that share over 45% of sequence identity. Recent evidence suggests that members of this wide-spread protein family can function both at the transcriptional and translational level in vitro. However, the exact mode of action has yet to be established. In addition, post-transcriptional regulation seems to play a major role in the induction of CSPs, a process in which the ribosome may be involved. This is in accordance with a model in which the ribosome has been proposed to be the sensor of temperature in bacteria.

Homologues of CspA are present in a number of bacteria. Widespread distribution, ancient origin, involvement in the protein translational machinery of the cell and the existence of multiple families in many organisms suggest that these proteins are indispensable for survival during cold-shock acclimation and that they are probably also important for growth under optimal conditions (Phadtare *et al*; 1999).

In contrast to stress conditions like heat shock, no cold-specific σfactor has so far been identified, suggesting that the cold shock response is predominantly post-transcriptional. Consistent with this notion, many of the proteins up-regulated in *E. coli* during cold shock are associated with the translational apparatus, such as the trigger factor, a ribosome-binding protein chaperone, CsdA, an ATP-dependent RNA helicase-like protein, RbfA, an RNA-binding protein implicated in ribosome assembly, and the translation initiation factors IF1 and IF3 (Wilson and Nierhaus ,2004).

2.8. Effect of cold shock in the release of toxins:

Survival and tolerance at cold temperatures, the differentially expressed cellular proteins, and cholera toxin (CTX) production were evaluated in *Vibrio cholerae* O1. Rapid loss of culturability and change to distinct coccoid morphology occurred when cultures of *V. cholerae* O1 were exposed to 5°C directly from 35°C. Also, cultures of *V. cholerae* first exposed to 15°C for 2 hours and then maintained at 5°C failed to exhibit an adaptive response, instead a rapid loss of viable plate count was noticed.

Results from Western blot experiments revealed the absence of a major cold shock protein, CS7.4. Also, a decreased level of CTX was noticed in *V. cholerae* O1 cultures exposed to 5°C or 15°C after first being exposed to 15°C for 2 hours, followed by transfer to 5°C. Reduced expression of CTX at cold temperatures, compared to the cultures maintained at 35°C, may be a result of decreased cellular metabolic activity. (Carroll, *et al*; 2001).

2.10. Effect of cold shock in the release of U.V absorbing materials:

Gross damage is indicated by leakage of U.V absorbing materials from the cell or increased access of molecules to the cytoplasmic compartment. Leaked materials include K^+, inorganic phosphate, phosphorylated sugars, sugars, fatty acids, esters and aminocompounds. An impression of the extent of injury was obtained from the size of molecules able to pass across the membrane. The cytoplasmic enzyme glucose 6- phosphoate dehydrogenase was unable to escape from the freeze –injured *E.coli* where as molecules of molecular mass 12KD (ribonuclease) but not 16KD (dextran) where able to enter the cell from out side (Lund *et al*; 2000).

Freezing and storage of *Escherichia coli* at –20 $^{\circ}$C in phosphate buffer resulted in loss of cell viability and a pronounced leakage of cellular material which had maximal absorption at 260 mμ. Greater loss in cell viability

occurred when cells were frozen in distilled water, but only small amounts of 260 mμ absorbing material were detected.

Unfrozen cells stored at 2˚C and 22˚C in each menstruum showed little loss in viability, but cells in phosphate buffer released significant amounts of material during storage. Leakage material from cells in phosphate buffer contained greater amounts of ribonucleic acid and amino acids than did material from cells in distilled water. Leakage material from frozen cells contained protein in the form of peptides of relatively small molecular weight; this was not observed for unfrozen cells. These compounds protected a dilute cell suspension from the lethal effects of freezing, and also possessed biological activity for the recovery of cells which had been "injured" by freezing. Direct cell counts indicated that the material released was not a result of cell lysis (Moss and Speck, 1966).

2.11. Repair of injured cells:

Hideharu (2006) define repair as the ability of injured cells to return to normal physiological function. Janssen and Busta (1973) reported that fast freezing and slow thawing of *Salmonella anatum* cells in nonfat milk solids resulted in about 20% death and 50% injury of the cells surviving the treatment.

Death was defined as the inability to form colonies on a non-selective plating medium xylose-lysine-peptone agar (XLP) after freezing and thawing. Injury was defined as the inability to form colonies on selective plating medium (XLP with 0.2% sodium desoxycholate added).The injured cells repaired rapidly and within 2 hr at 25 °C, in the presence of 0.1% milk solids, all the injured cells regained the ability to form colonies on the selective medium.

The treated cells showed a 1-hr extended lag phase of growth as compared to the unfrozen cells. Milk solids concentration in the freezing and repair menstrua influenced injury, repair of injury and death.

The same above investigators concluded that, the repair process was affected by the pH and temperature of environment in which the injured cells were incubated. Maximum repair occurred at pH values between 6.0 and 7.4 and temperatures from 25°C to 42°C. The data suggested repair did not require the synthesis of protein, ribonucleic acid, or cell-wall mucopeptide but did require energy synthesis.

After *Escherichia coli* was injured by freezing, the repair process was studied during incubation of the cells for 2 hours at 25 °C in 0.5% K2HPO4 at pH 7.0 in the presence of specific metabolic inhibitors.. Data indicated that the cells synthesized energy in the form of ATP and probably utilized it for the repair process. Addition of ATP also facilitated the repair of injury. The freeze-injured cells showed extreme susceptibility to surface-active agents and lysozyme. The repaired cells, like the uninjured cells, became relatively resistant to these compounds. (Ray and Speck, 1972).

2.12. The effect of selective media on recovery of injured bacterial cells:

When Morichi and Irie (2004) investigated conditions facilitating recovery of sublethally injured cells present in frozen or freeze-dried preparations of bacteria, they noted that some cells of *Streptococcus faecalis* injured by freezing required RNA resynthesis for recovery, whereas the rest did not. Addition of 6% NaCl to control recovery medium was found to inhibit RNA synthesis completely in frozen-injured cells of *Streptococcus faecalis*, but not in intact ones. This seems to be a principal reason why 6% NaCl inhibits the growth of the injured cells.

The same investigators reported that the recovery of viable cells of freeze-dried and stored *Streptococcus thermophilus* was greatly increased by the addition of cysteine to the plating medium. The favorable effect of cysteine was mainly due to its reducing activity. On the contrary, the addition to the plating medium of certain types of peptone, such as lactalbumin hydrolyzate, resulted in a considerable decrease of viability in frozen preparations of *Vibrio metschnikovii*.

Ray and Speck (1972) recorded that Freezing an aqueous suspension of *Escherechia coli* NCSM at –78°C for 10 min, followed by thawing in water at 8°C for 30 minute , resulted in the death of approximately 50% of the cells, as determined by their inability to form colonies on Trypticase soy agar containing 0.3% yeast extract (TSYA). Among the survivors, more than 90% of the cells were injured, as they failed to form colonies on TSYA containing 0.1% deoxycholate.

They also concluded that injury was reversible as the injured cells repaired in many suitable media. The rate of repair was rapid and maximum in a complex nutrient medium such as Trypticase soy broth supplemented with yeast extract.

2.12. The pathogenecity of injured cells:

In (1990) Macfadyen stated that "pathogenic bacteria probably do not lose their virulence by freezing".

Freezing at –75°C and storage at–20°C of a cell suspension of *Salmonella gallinarum* resulted in a heterogeneous population of dead, metabolically injured, and unharmed cells. Injured cells constituted as much as 40% of those surviving freezing and storage for one day. Replica plating of frozen and thawed cells indicated metabolic injury was repairable and not a stable mutation.

Penicillin was used to increase the ratio of injured to uninjured cells from a frozen and thawed cell suspension. Pathogenicity was evaluated by observing percent mortality after injecting injured or uninjured cells into separate sets of chicks.

Mortality differences between wholly uninjured and predominantly injured populations were small and consistent (5% level) with a hypothesis of no difference. (Sorrels *et al*; 1970)

As in many bacteria, including such pathogens as *Salmonella enteritidis*, enterotoxic *Escherichia coli, Vibrio vulnificus, Vibrio cholerae*, the occurrence of a viable but non-culturable (VBNC) state has been described for *Campylobacter jejuni*. This bacterium has been recognized as a leading foodborne pathogen. VBNC cells from three *Campylobacter. jejuni* human isolates were suspended in microcosm water at 4°C and entered the VBNC state.

The metabolic activity of these VBNC cells was monitored by CTC reduction. Once in VBNC state, the strains were inoculated per os into newborn mice and 1-day-old chicks so as to evaluate and compare these animal models of VBNC cell recovery.

The three strains used were revived using the murine model, whereas only two strains were revived with the chick model.

The results showed that the murine model permits better recovery than the 1-day chick model. All three strains revived exhibited an associative index very similar to that measured in the culturable state. This study indicates that the VBNC state should be considered as playing a role in the epidemiology of *Campylobacter* infection. (Cappelier *et al*; 1999).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Organisms:

The organisms used in this study included *Staphylococcus aureus*, *Escherichia coli, Salmonella spp*. and these were obtained from the stock culture of the department of microbiology- faculty of veterinary medicine - University of Khartoum. *Pasteurella multocida* type B which was used to study the pathogenecity of injured cells was supplied by the biological products department- central veterinary research laboratories center, Soba.

3.2 Media:

The following media were used; it was obtained in dehydrated form and was rehydrated as described by the manufacturers.

3.2.1. Nutrient broth (Oxoid CM1):

Thirteen grams of dehydrated (oxoid) nutrient broth were suspended in a liter of distilled water, mixed well and the pH adjusted to 7.4 and then sterilized by autoclave at 121˚C for 15 minutes.

3.2.2. Nutrient agar (Oxoid CM3):

It consists of (grams per liter) lab-lemco powder 1.0 gram, yeast extract 2 grams, peptone 5 grams, sodium chloride 5 grams and agar 15 grams. Twenty-eight grams of the dry medium were added to 1 liter of distilled water and boiled to dissolve completely, the pH was adjusted to 7.4, and then the medium was sterilized by autoclaving at 121º C for 15 minutes and distributed aseptically in 15 ml amounts into sterile Petri dishes.

Two types of media were used for the viable counts and enumeration of the organisms:

A. None selective media:

a) Plate count agar which was used for the viable count of *Staphylococcus aureus, Escherichia coli* and *Salmonella spp*.

b*)* Blood agar was used for *Pasteurella multocida* type B.

a) Plate count agar (Oxoid CM325) consists of g/l (w/v): 15 yeast extract, 5 pancreatic digest casein, 1 glucose and 15 agars. The ingredients were added to one liter of distilled water and completely dissolved by boiling. The pH was adjusted to 7.2 the media then autoclaved at 121˚C for 15 minutes and then poured on plates. .

b) Blood agar (Oxoid CM55):

Forty grams of blood agar base were suspended in a liter of distilled water, steamed to dissolve, cooled and the pH adjusted to 7.4. It was then sterilized by autoclaving at 121˚C for 15 minutes .After cooling to about 45˚C, sheep blood was added aseptically in 7-10% concentration before pouring plates.

B. Selective media:

1. Baird - parker media (Oxoid CM275) was used for *Staph. aureus.* In a liter of distilled water, 63 grams of dehydrated (oxoid) Baird- parker medium were suspended, mixed and steamed to dissolve, the pH was adjusted to 6.8 before autoclaving at 121˚C for 15 minutes. After cooling to about 45˚C, five ml of egg- yolk emulsion and two ml of potassium tellurite were added aseptically and mixed well before pouring on to the plates.

2. MacConkey agar (**(Oxoid CM109**) was used for enumeration of *E.coli* and *Salmonella spp*. It consisted of (g/L) peptone 20, lactose 10, bile salts 5, neutral red 0.075, agar 12, and sodium chloride 5. The pH was adjusted to 7.4.

3. Pasteurella medium (Sayda, 1993). This was used to culture the *Pasteurella multocida* type B then different dilutions were made from this

medium before and after freezing *Pasteurella multocida*. The medium consisted of protease peptone 15g ,yeast extract 5g ,glucose 2g ,sucrose 2.5g , sodium sulphate(Na_2So_3) o.2g , cysteine o.5g , potassium dihydrogen sulphate(KH_2 PO_4) 4g. All ingredients (except cysteine) were dissolved in liter of distilled water.

Two hundred micro liter of hydrochloric acid 37% was added to cysteine to dissolve it and it was put in water bath for 10 minutes before being added to other ingredients of the medium. The pH was adjusted to 7.4. Then the medium was dispended in to 100 ml bottles and autoclaved at 121˚C for 15 min.

4. Pasteurella solid medium:

This media has the same ingredients as above plus 2% agar. It was used to determine the dilutions that contained injured cells by comparing growth of different dilutions on this medium with growth of different dilutions on blood agar after freezing.

3.3. Normal saline solution:

This was prepared by dissolving 8.5 gram of sodium chloride in 1 liter of distilled water (Cowan and Steel, 1985).

The *E.*coli and the *Salmonella spp*. were subcultured from slopes on MacConkey agar, and *Staph. aureus* on blood agar and incubated at 37˚C for 18hr. Then they were subcultured on nutrient agar and incubated at 37˚C for 18hr. After that the organisms were subcultured on nutrient broth and incubated at the same above temperature for the same time. These were used for viable counts procedures.

Colonies from pure culture of *Pasteurella multocida* type B were transferred to 100 ml bottle containing Pasteurella liquid medium and incubated at 37˚C over night. For purity Gram stain smear was done.

3.4 Viable count:

For viable count the method of Miles and Misra was used (Miles and Misra 1938). The procedure of this method was as follows:

One ml of an 18 hr culture was added to one of the test tubes containing 9ml of the diluent i.e normal saline solution ,then serial ten- fold dilutions were made and 0.1ml of appropriate dilutions were pippeted onto dry surface of the Petri dishes containing the selective and non- selective media. The surfaces were well spreaded using sterile bent glass rods and incubated at 37˚C for 24 hour. The number of the viable bacteria per 1ml was equal to the number of the colonies per plate multiplied by 10 multiplied by the dilution factor. The result was recoded as the number of colony forming units per ml (C.F.U/ ml).

Colonies were counted and these were recorded as the initial viable count. The cultures were then stored at deep freezer adjusted to temperature of –20˚C for 24 hour.

The cultures were removed from the deep freezer and held at the room temperature until thawed. The number of the viable cells was enumerated on the selective and non- selective media. For *Pasteurella multocida* type B, to determine the dilutions that contain injured cells, growth of different dilutions on Pasteurella medium was compared with growth of different dilutions on blood agar after freezing. The dilutions that showed growth on blood agar but not on Pasteurella medium are the dilutions that contained injured cells.

To determine the dilutions that contained dead cells, growth of different dilutions on blood agar after freezing were compared with growth of different dilutions on blood agar before freezing. Dilutions that showed

growth on blood agar before freezing and not after freezing are the dilutions that contained dead cells.

Injury was determined by the number of cells on the non- selective recovery media minus the number of cells on the selective media.

Dead cells were determined by the number of cells before freezing on non-selective media minus the number on selective media after freezing (Patterson and Jackson, 1979).

3.5 Injection of the rabbits:

Five local breeds' rabbits were used to study the pathogenecity of injured cells of *Pasteurella multocida* type B .

Several tubes from dilutions that contained injured cells were concentrated by centrifugation at 4˚C at 4000 rpm for 20 minutes. Most of the supernatant were discarded and about 2 ml from each dilution were left. The deposits were pooled.

To be sure these cells were injured and not alive cells, 0.1ml from pooled supernatant was spreaded on both blood agar and pasteurella media and incubated at 37 ºC overnight.

Three melliters of injured cells were injected intramuscularly in each of the four rabbits. The fifth rabbit was injected with 3 ml of dead cells that were prepared similar to injured cells.

3.6 Effect of freezing of bacterial cells on the release of ultra-violet absorbing materials:

Eighteen hour old cells of *Staph. aureus, E coli* and *Salmonella spp* which were cultured on nutrient broth or Pasteurella medium, for *Pasteurella multocida* type B, were centrifuged at 4000 rpm for 15min. using (HETTIC – MIKRO22) cold centrifuge(4˚C). Then the same cultures were stored at – 20˚C for 24 hr. After thawing at room temperature the treated cells were

centrifuged using the previous centrifuge.Both before freezing and after freezing cultures were read by spectrophotometer (JASCO V-530), at 280n.m.

CHAPTER FOUR
RESULTS

- The result of viable count of *E.coli*, on selective (MacConkey' s agar) and non- selective Plate count agar(P.C.A) media before and after freezing at –20˚C and percentage of injured, uninjured and dead cells are shown in **Table (1).**
- The results of viable count from the dilutions 10^{-1} to 10^{-5} showed uncountable colonies according to the three organisms tested.

Table (1): viable count before and after freezing of *E.coli*:

Enumeration media	Media	CFU/ml		**%of injury**	**%of Death**	**%of uninjured**
		Before freezing	After freezing			
P.C.A	Non-selective	4.7×10^8	2.1×10^8	**14.8%**	**55.4%**	**29.8%**
MacConkey agar	selective	7.3×10^8	1.4×10^8			

- Injury was determined by the number of cells on the non- selective recovery media minus the number of cells on the selective media.
- Death was determined by the number of cells before freezing on non-selective media minus the number on non-selective media after freezing.
- Uninjured was determined by the number of cells on the selective media after freezing divided on the number of cells on non- selective media before freezing.

- The result of viable count of *Salmonella* spp. on selective and non-selective media before and after freezing at -20˚C and percentage of injured, uninjured and dead cells are shown in **Table (2)**.

Table 2: viable count before and after freezing of *Salmonella* spp*:*

Enumeration media	Media	CFU/ml		**% of injury**	**% of Death**	**% of uninjured**
		Before freezing	After freezing			
P.C.A	Non-selective	1.2×10^9	4.7×10^8	**18.5%**	**61. %**	**20.5%**
MacConkey agar	selective	1.8×10^9	2.5×10^8			

- The results of viable count of *Staphylococcus aureus* on selective and non- selective media before and after freezing at -20˚C and percentage of injured, uninjured and dead cells are shown in **Table (3).**

Table (3): viable count before and after freezing of *Staphylococcus aureus:*

Enumeration media	Media	CFU/ml		**% of injury**	**% of Death**	**% of uninjured**
		Before freezing	After freezing			
P.C.A	Non-selective	1.81×10^9	4.9×10^8	**11%**	**72.%**	**16.1%**
Baird-Parker media	selective	5.2×10^8	2.9×10^8			

Table (4): The dilutions of *Pasteurella multocida* type B that showed no colonies on selective media but showed colonies on blood agar.

The dilutions	Viable count (C.F.U/ml)
10^{-1}	Uncountable colonies
10^{-2}	Uncountable colonies
10^{-3}	Uncountable colonies
10^{-4}	1.93×10^{7}

- The dilutions from 10^{-5} to 10^{-10} showed no colonies neither on blood agar nor on Pasteurella medium (dead cells).

Results of injection of rabbits by freeze- injured cells to study their pathogenecity:

There was no growth of the dilutions inocualted on Pasturella media while there was growth on blood agar (injured cells).

The first four rabbits died with in 24 hour after showing respiratory signs, and the result of postmortem, under aseptic conditions, showed hearts and lungs congestion. The plates inoculated with the blood aspirated from the hearts showed rich growth of *Pasteurella multocida* colonies which have characteristic odour.There was obvious turbidity in the nutrient broth tubes. The blood films which were stained with Gram's stain showed small, Gram (–) negative coccobacilli.

The fifth rabbit was under monitoring for 5 days before released. There was no growth of any kinds of colonies on blood agar which inoculated by blood from the ear vein of this rabbit. The rabbit was released in the sixth day.

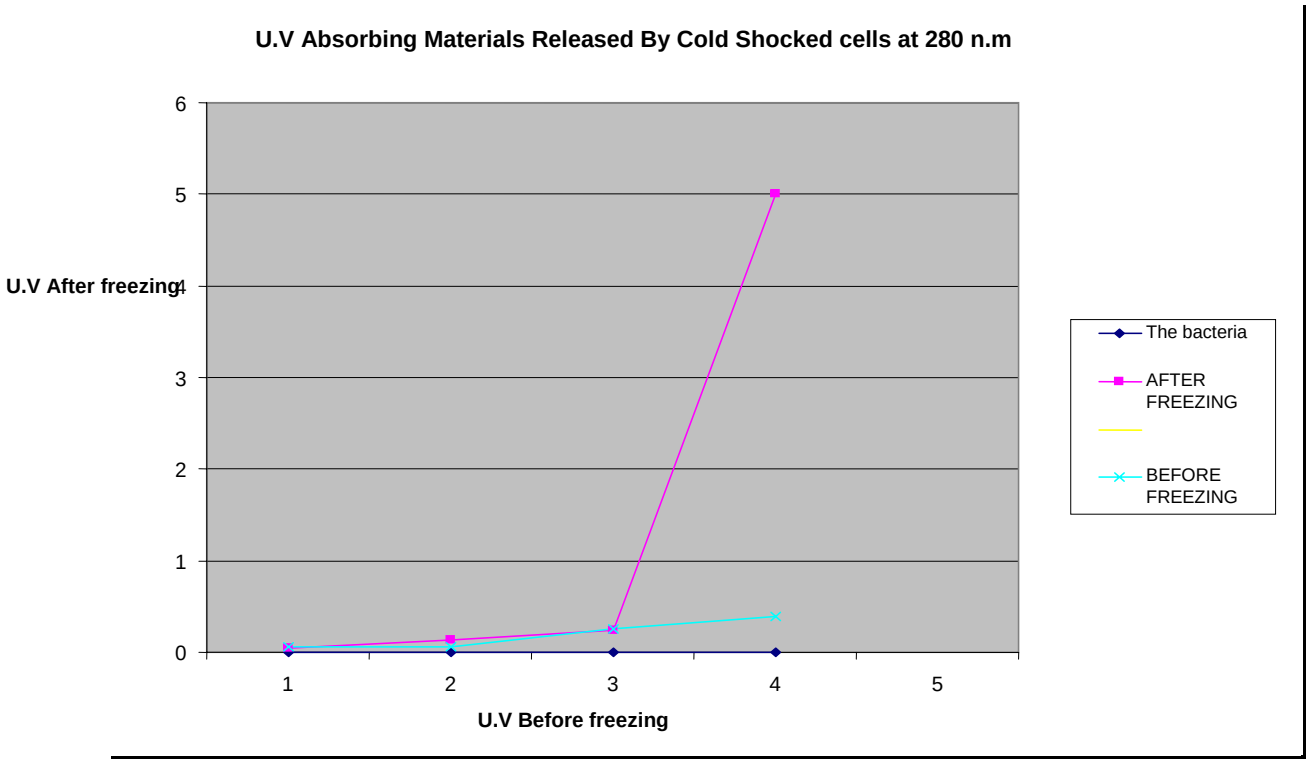

Figure 1: The release of U.V absorbing materials at 280 n.m after freezing the four organisms at –20˚C.

1. *Staph. Aureus.*
2. *Salmonella spp.*
3. *E.coli.*
4. *Pasteurella multocida* type B.

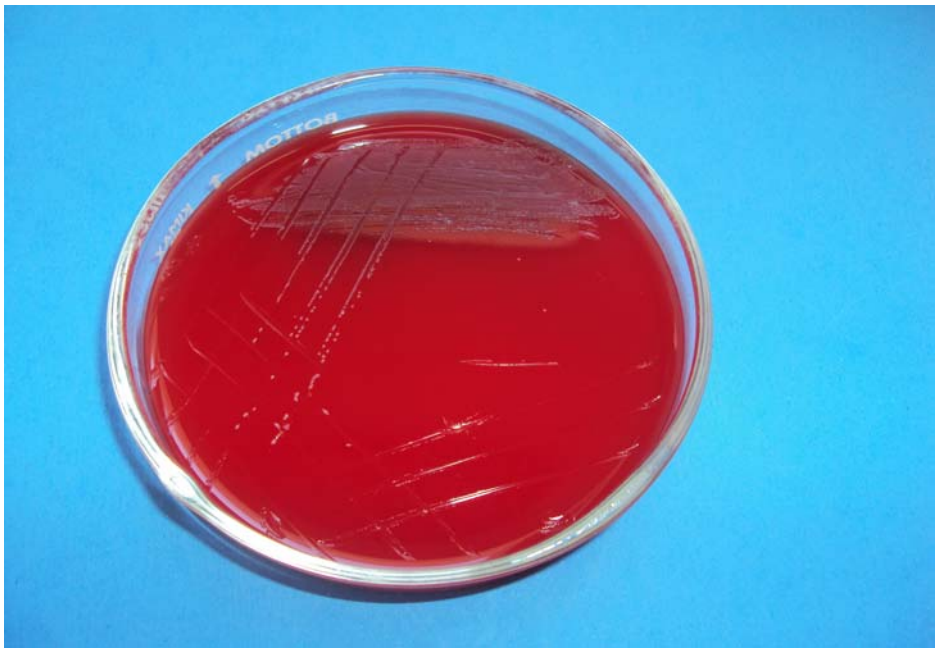

Figure (2): Growth of *Pasteurella multocida* type B on blood agar after isolation from aspirated blood from the first rabbit's heart after death.

Figure (3): Growth of *Pasteurella multocida* type B on blood agar after isolation from aspirated blood from the second rabbit's heart after death.

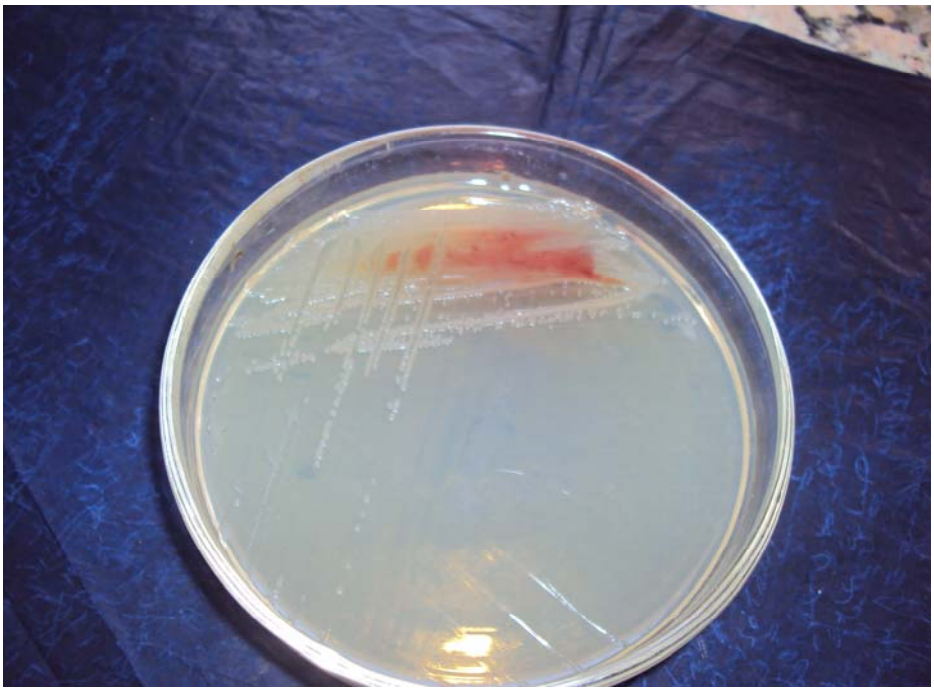

Figure (4): Growth of *Pasteurella multocida* type B on Pasteurella media after isolation from aspirated blood from the first rabbit's heart after death.

Figure (5): Growth of *Pasteurella multocida type* B on Pasteurella media after isolation from aspirated blood from the second rabbit's heart after death.

CHAPTER FIVE

DISSCUSION

When bacteria are frozen and subsequently thawed three categories of cells can be distinguished: un injured, injured and killed. Uninjured cells are capable of growth on minimal nutritive media or on the selective media normally used in their isolation, conversely, killed cells would be unable to grow on any medium. Injured cells are nutritionally more demanding whilst repair of freezing – induced injury is being affected, they only grow on media which provide certain energy- requiring factors necessary for the repair of the injury. This repair is rapidly being completed in less than 2 hours, it can be performed by cells in the thawed out food providing the required nutrients are available (Forsythe, 1998).

In this study, the evaluation of the effect of freezing and thawing on *E.coli* and *Salmonella spp*. showed that they are susceptible to freezing and this is in line with previous findings (Moss and Speck, 1966; Layla, 1987 and Digirolamo, 2006).

The responses of the organisms tested varied from one to another and this agreed with Kraft (1992) who recorded that the responses of the organisms to freezing varies considerably , some are killed and some survive and may remain viable to different degrees during frozen storage and after thawing.

The colonies that developed on non-selective medium represent both injured and un injured cells whereas only the un injured cells developed on the selective media.

Acorrding to *Salmonella spp* 61% of the cells died and failed to form colonies neither on plate count agar (non- selective media) nor on MacConkey's agar (selective media). 18.5% of the survivors failed to form

colonies on the selective media because of their injury, but they formed colonies on non- selective media.

The rest of the cells (20.5%) were uninjured as they grew on selective and non- selective media. These results are similar to these obtained by Janssen and Busta (1973) as they reported that fast freezing and slow thawing of *Salmonella anatum* cells in nonfat milk solids resulted in about 20% death and 50% injury of the cells surviving the treatment. Death was defined as the inability to form colonies on a nonselective plating medium (xylose-lysine-peptone agar (XLP) after freezing and thawing. Injury was defined as the inability to form colonies on selective plating medium (XLP with 0.2% sodium desoxycholate added). The injured cells repaired rapidly and within 2 hours at 25 °C, in the presence of 0.1% milk solids; all the injured cells regained the ability to form colonies on the selective medium.

Death of *E.coli* cells from freezing at –20°C was 55.4%, 14.8% of the cells were injured and 29.8% were uninjured cells .These results substantiated the findings of Ray and Speck (1972) who found that freezing an aqueous suspension of *Escherichia coli* NCSM at –78 °C for 10 min, followed by thawing in water at 8°C for 30 min, resulted in the death of approximately 50% of the cells, as determined by their inability to form colonies on Trypticase soy agar containing 0.3% yeast extract (TSYA). Among the survivors, more than 90% of the cells were injured, as they failed to form colonies on TSYA containing 0.1% deoxycholate.

The injury of *Pasteurella multocida* type B was obtained by freezing at –20°C and thawing at room temperature and this in line with Straka and Stokes (1959) who observed injury in *E.coli* and *Pseudomonas species* after freezing storage. The growth of the organism on blood agar and their failure to grow on the selective media indicated that they lost their colony forming

ability and this agreed with the definition of injured cells by Clark and Ordal (1969) who defined the injured cells are those cells which can form colonies on enriched media but can not form colonies on stressing media.

The death of the four rabbits after 24 hour of injection by injured cells of *Pasteurella multocida* type B, suggests that injury due to freezing did not alter pathogenecity and these finding are in accordance with that of Simon *et al* ;(1963) who found that *Salmonella typhymuirium* remained virulent for mice after being freeze- dried and stored 1-2 years at 5˚C. Also Szturn-Rubinsten *et al* ;(1969) reported that *Shigella Sonneii* frozen in ice and used in patient's food was responsible for dysentery. The inoculation of blood aspirated from the hearts of the dead rabbits, on Pasteurella selective media and the rich growth resulted from that inoculation indicated that repair of injury occurred and this is in agreement with Sorrells *et al;* (1970) who concluded that *Salmonella gallinarum* freeze- injured cells, after injection in chick embryo repaired it self and this repair may have occurred while the cells were with in the intraperitoneal cavity.

The survival of the fifth rabbit which was injected with cells that failed to form colonies neither on Pasteurella selective media nor on blood agar and this suggested that these were dead cells and this findings confirms that of Janssen and Busta (1973) as they reported that death was defined as the inability to form colonies on a non-selective plating medium after freezing and thawing. Also the result of failure of growth obtained from the inoculation of blood from the ear vein of the same rabbit supports that suggestion.

The results of freezing the four organisms at –20˚C and release of u.v absorbing materials at 280 n.m simulated the results obtained by Moss and Speck (1966) who reported that freezing and storage of *Escherichia coli* at

–20 ˚C in phosphate buffer resulted in loss of cell viability and a pronounced leakage of cellular material which had maximal absorption at 260 n.m.

Also our results substantiated the findings of Ray and Speck (1972) who reported that freezing an aqueous suspension of *Escherichia coli* NCSM at –78 ˚C for 10 min., followed by thawing in water at 8˚C for 30 min. resulted in death and injury which was accompanied by the loss of 260 n.m and 280 nm absorbing materials from the intracellular pool.

Staph. aureus in this study showed the highest percentage of death (72.9%),but the injured cells were 11% and the uninjured cells were 16.1% and this result is in contrast with Ray(1989) who recorded that Gram-negative organisms are more susceptible to injury than Gram positive organisms. While this is so, more work is warranted to study the effect of cold storage on many bacteria, the mechanism of the phenomenon and the best mean to resuscitate such cells.

CONCLUSIONS

- Injured bacteria failed to form colonies on selective media but formed colonies on non-selective media.
- The effect of freezing in bacteria varied in diffrent organisms, some are very sensitive as *Pasteurella multocida* type B while others as *E.coli* are less sensitive.
- The injury which resulted from freezing was repairable and the injection of injured *Pasteurella multocida* type B cells caused rabbits death in 24 hour.
- There was slight increase in the concentration of the u.v absorbing materials released by the cells at 280 n.m.before and after freezing at –20˚C.

RECOMMENDATIONS

It is recommended that:

- More studies should be done on the nature of cellular damage at the molecular level in injured bacteria and the mechanisms of repair of injury.
- The most effective methods to allow enumeration and isolation of injured bacteria from frozen food samples because they are not effectively detected either by selective or non-selective media due to reversible injury.

CHAPTER SIX

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